SUPPLEMENTARY MATERIAL

Crystallographic analysis of human hemoglobin elucidates the structural basis of the potent and dual antisickling activity of pyridyl derivatives of vanillin

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Synopsis: Pyridyl derivatives of vanillin increase the fraction of the more soluble oxygenated sickle hemoglobin and/or directly increase the solubility of deoxygenated sickle hemoglobin. Crystallographic analysis reveals the structural basis of the potent and dual antisickling activity of these derivatives.

1. Detailed biochemical experimental procedures

1.1. *In vitro* sickling of SS cells and morphological analysis.

Sickling experiments were conducted as previously reported (Abdulmalik *et al*, 2005). Briefly, suspensions of SS cells in Hemox buffer, pH 7.4, containing 10 mM glucose and 0.2% bovine serum albumin were pre-incubated under air in the absence or presence of three different concentrations (0.5, 1, and 2 mM) of INN-312 and INN-298 or vanillin at 37^{o}C for 30 min. The suspensions were subsequently incubated under 4% oxygen/96% nitrogen at 37° C for 5 hrs. Aliquots (5 μL) of each sample were drawn into a fixing solution (2% glutaraldehyde) where they were instantaneously fixed under identical low oxygen pressures to preserve their morphology. Subsequently, fixed cell suspensions were introduced into glass microslides and subjected to microscopic morphological analyses (Asakura & Mayberry, 1984; Horiuchi *et al*, 1990).

1.2. Determination of the oxygen equilibrium curves with sickle Hb.

The OECs of suspensions of SS cells and their corresponding hemolysates were determined with a Hemox™ Analyzer (TCS Scientific Corp.) as described elsewhere (Abdulmalik *et al*, 2005; Asakura, 1979). Aliquots of residual RBC suspensions treated with INN-312 and INN-298 or vanillin from the above sickling assay were utilized. Approximately 50 μl of each suspension was added to 4.5 ml of Hemox buffer, pH 7.4, in a cuvette and subjected to OEC analysis. The OECs were determined for stroma-free cell lysates in 0.1 M potassium phosphate buffer, pH 7.0, at 25° C.

1.3. Determination of Hb adduct formation with Sickle Hb.

The hemolysates that had been prepared from SS cells after incubation with test compounds were subjected to cation-exchange HPLC (Hitachi D-7000 Series, Hitachi Instruments, Inc., San Jose, CA), using a weak cation-exchange column (Poly CAT A: 50 mm x 4.6 mm, Poly LC, Inc., Columbia, MD), and eluted with a linear gradient of phase B from 25% to 90% at 410 nm (Mobile Phase A: 40 mM Bis-Tris, 5 mM EDTA, pH 6.5; Phase B: 40 mM Bis-Tris, 5 mM EDTA, 0.2 M sodium chloride, pH 6.5).

1.4. Determination of the effect of INN-312 on deoxy-Hb S solubility.

The solubility of deoxy-Hb S in 1.7 M phosphate buffer (pH 7.4) was measured using a modification of previously reported methods (Fabry *et al.*, 2003; Fabry *et al.*, 2001). Eight concentrations of Hb S (0.0025 - 0.05 g/dL) were prepared from lysates of RBCs and then incubated with 0, 0.5, 1 or 2 mM INN-312. Each reaction was prepared in quadruplicate to permit assay reproducibility. The samples were deoxygenated with 30 mM sodium dithionite, and then sealed with oxygen-impervious optically inert mineral oil. The samples (150 µl) were incubated at 30°C for 60 min in a 96-well 0.45 µm Multiscreen® Solvinert filter plate (Millipore Corp., Billerica, MA), and then vacuum-filtered into a collection plate where it was maintained under deoxygenated conditions. The absorbance of the filtrates (corresponding to soluble heterotetrameric Hb) was determined at 556 nm. The gelation concentration (C_{SAT}) of each individual Hb sample was calculated as the point where the concentration of the filtrate became lower than the input T state Hb concentration.

References

Abdulmalik, O., Safo, M.K., Chen, Q., Yang, J., Brugnara, C., Ohene-Frempong, K., Abraham, D.J. & Asakura, T. (2005). *British Journal of Haematology,* 128, 552-561.

Asakura, T. (1979). *Critical Care Medicine,* 7, 391-395.

Asakura, T. & Mayberry, J. (1984). *The Journal of Laboratory and Clinical Medicine,* 104, 987-994.

Horiuchi, K., Ohata, J., Hirano, Y. & Asakura, T. (1990). *The Journal of Laboratory and Clinical Medicine,* 115, 613-620.

Table S1

Crystallographic data and refinement statistics for the T State Hb complex structures

Values in parentheses refer to the outermost resolution bin

	INN-298	INN-312
Data collection statistics		
Space group	$P2_1$	$P2_1$
Cell dimensions (Å)	62.2, 82.9, 53.5; 99.5	63.3, 83.7, 53.9; 99.5
Molecules/asymmetric unit	1 tetramer	1 tetramer
Resolution (Å)	49.1-1.6 (1.66-1.60)	53.1-1.94 (2.25-1.94)
No. of measurements	199132 (13891)	143220 (46084)
Unique reflections	68265 (6941)	39097 (13554)
I/sigma I	23.8 (6.6)	15.3 (6.1)
Completeness (%)	98.0 (88.8)	92.7 (90.7)
Rmerge (%)[†]	3.2 (8.9)	7.3 (14.8)
Structure refinement		
Resolution limit (Å)	49.1 – 1.60 (1.67 - 1.60)	41.9-1.94 (2.03-1.94)
Sigma cutoff (F)	0.0	0.0
No. of reflections	68240 (7469)	39078 (4389)
Rfactor (%)	20.1 (27.1)	16.5 (18.7)
Rfree (%)[‡]	21.3 (31.0)	19.4 (22.2)
Rmsd standard geometry		
Bond-lengths (Å)/ -angles (°)	0.007 / 1.5	0.007/ 1.4
Dihedral angles		
Most favored /allowed regions	88.9/6.6	93.4/6.0
Average B-Factors		
All atoms	14.9	23.0

[†]Rmerge = $\Sigma_{hkl}\Sigma_i|I_{hkli} - \langle I_{hkli}\rangle|/\Sigma_{hkl}\Sigma_i\langle I_{hkli}\rangle$. [‡]Rfree calculated with 5% of excluded reflection from the refinement.

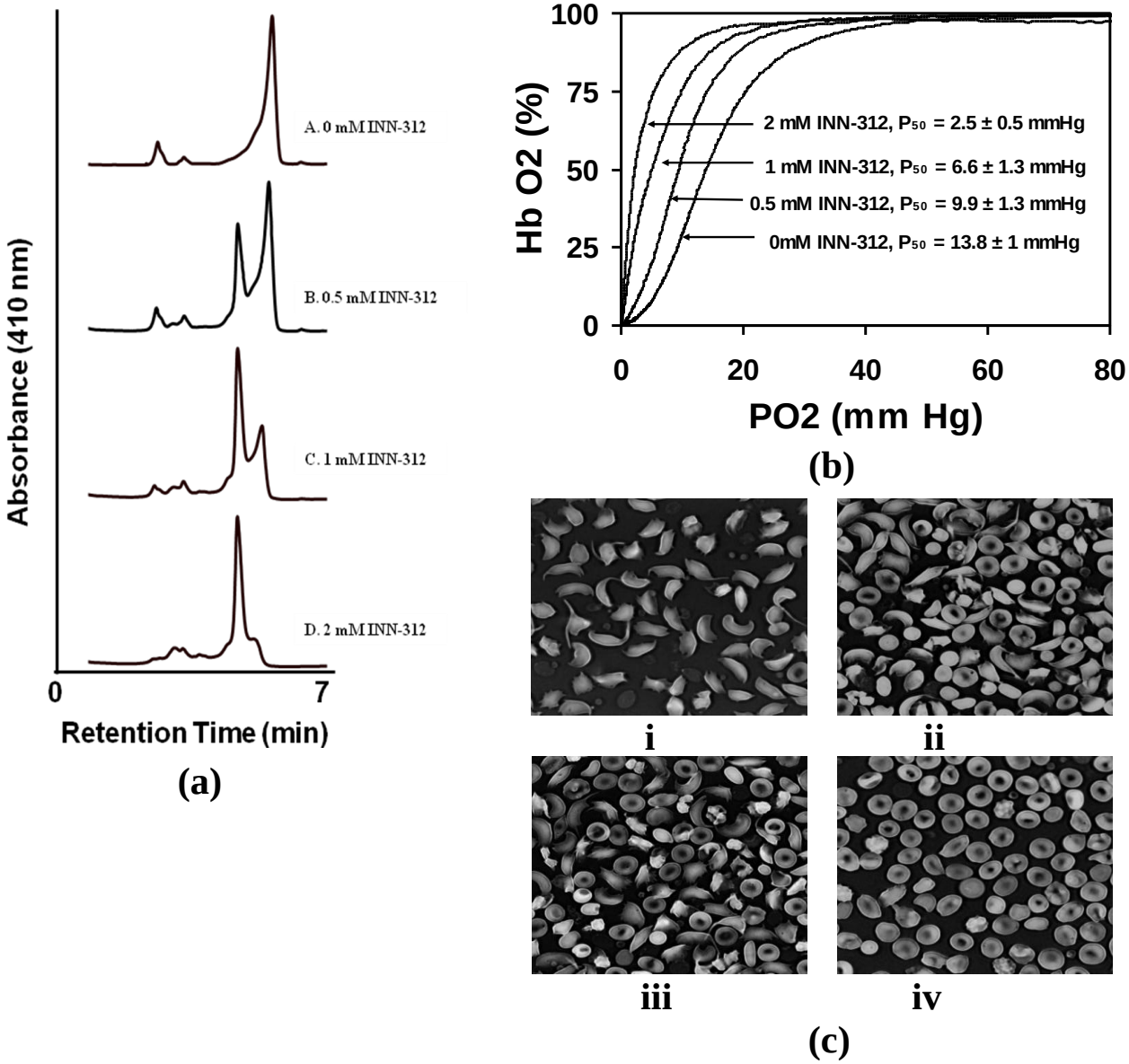

Figure S1. (a) Cation-exchange HPLC patterns of Hb prepared from SS cells that had been pre-incubated with 0 to 2 mM INN-312. Note that INN-298 also shows similar result. **(b)** OECs of

Hb S after hemolyzing SS cells after pre-incubation in the presence of 0 to 2 mM INN-312. **(c)** Morphology of SS cells after incubation with various concentrations of INN-312 under 4% oxygen at 37 °C for 5 h. **(i-iv)** In the absence of INN-312, and presence of 0.5, 1 or 2 mM INN-312, respectively. At the onset of the experiment, SS cells were almost exclusively normal (round, discoid), with the exception of a few irreversibly sickled cells. During hypoxia, more than 90% of untreated RBCs underwent sickling after 5-hour incubation [Figure 2c(i)], while the presence of INN-312 significantly reduced the percentage of sickled cells in a concentration-dependent manner [Figure 2c(ii-iv)].

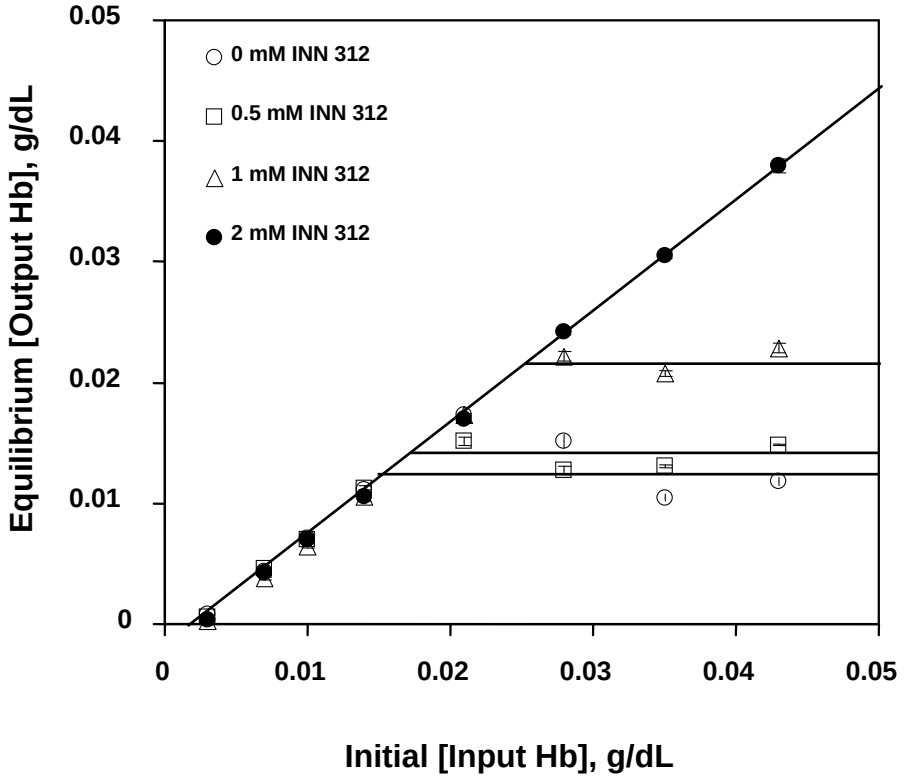

Figure S2. Measurement of deoxy-Hb S solubility in the absence and presence of 0.5, 1, or 2 mM INN-312. Shown are the concentrations of soluble deoxy-Hb S (y-axis) relative to the initial input T state Hb (x-axis). The gelation concentration of Hb (Csat) was determined as the concentration when the soluble Hb becomes lower than the input T state Hb concentration. The points of intersection of the horizontal lines with the linear represent the approximate Csat values for Hb bound with or without varying concentrations of INN-312. Each reaction was prepared in quadruplicate to permit assay reproducibility.

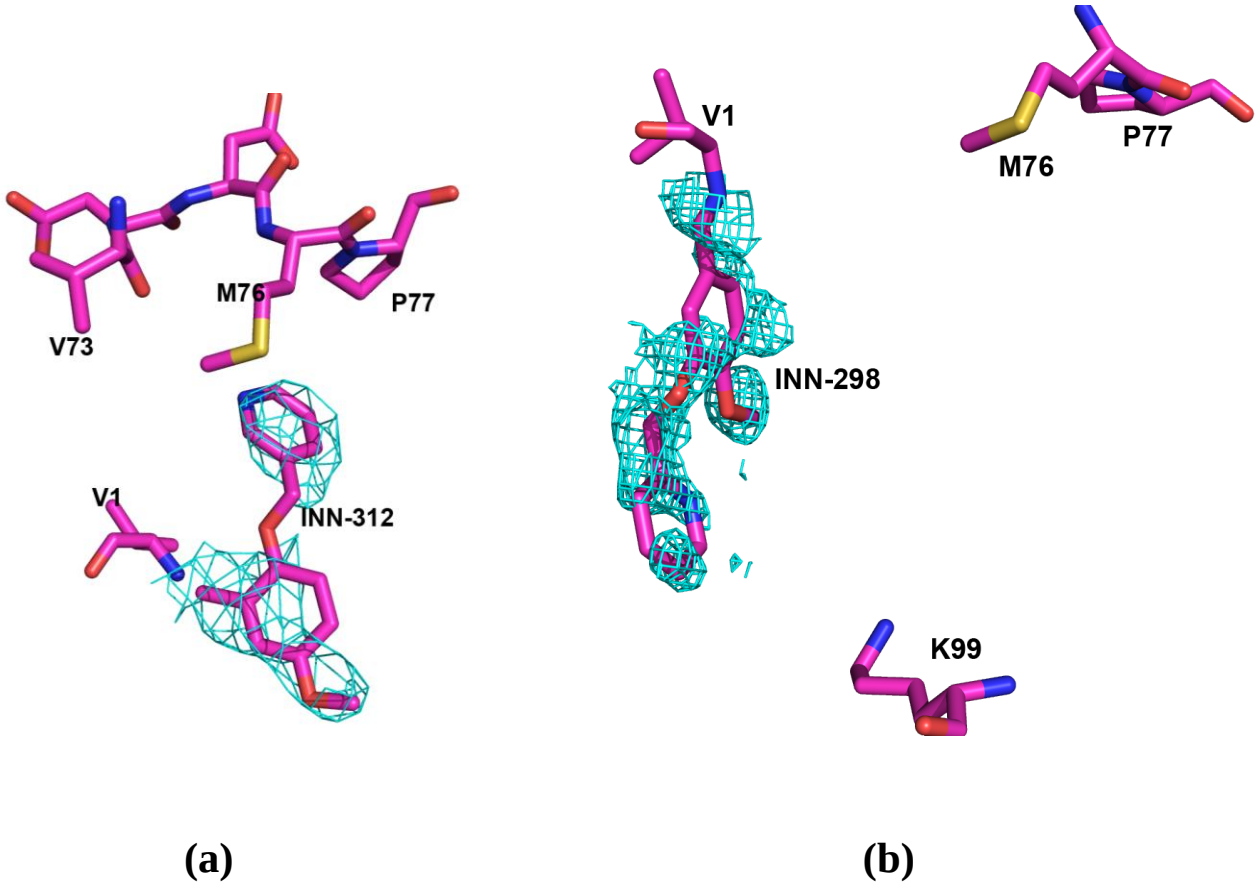

Figure S3. Difference electron-density maps for the T state complexes of INN-312 at 2.2 Å **(a)** and INN-298 at 2.0 Å **(b),** computed from Fobs(derive) – Fobs(native) amplitudes (at the 1.8σ level), where Fobs(derive) and Fobs(native) are structure factors from the complex crystals, and the previously determined structure of native T-state Hb (Fermi et al., 1984; PDB code 2HHB), respectively. Only one of the two bound molecules are shown. As noted in the manuscript, refinement of the INN compound positions did not improve the densities, and even became worse.